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Streptococcus mutans: Fructose Transport, Xylitol Resistance, and Virulence

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Abstract

Streptococcus mutans, the primary etiological agent of human dental caries, possesses at least two fructose phosphotransferase systems (PTSs), encoded by *fru*I and *fru*CD. *fru*I is also responsible for xylitol transport. We hypothesized that fructose and xylitol transport systems do not affect virulence. Thus, colonization and cariogenicity of *fru*I^{-} and *fru*CD^{-} single and double mutants, their WT (UA159), and xylitol resistance (X^{r}) of *S. mutans* were studied in rats fed a high-sucrose diet. A sucrose phosphorylase (*gtf*A^{-}) mutant and a reference strain (NCTC-10449S) were additional controls. Recoveries of *fru*I mutant from the teeth were decreased, unlike those for the other strains. The *fru*CD mutation was associated with a slight loss of cariogenicity on enamel, whereas mutation of *fru*I was associated with a loss of cariogenicity in dentin. These results also suggest why xylitol inhibition of caries is paradoxically associated with spontaneous emergence of so-called X^{r} *S. mutans* in habitual human xylitol users.

Keywords

Streptococcus mutans; PTS; xylitol; sucrose phosphorylase; caries

INTRODUCTION

Analysis of both experimental animal and human data indicates that maximal expression of dental caries is associated with both a diet rich in sucrose, consumed at high frequency, and colonization of the teeth by mutans streptococci (Mandel, 1970; Tanzer, 1979; Kuramitsu, 1993; Tanzer *et al.*, 2001a).

The roles of sugar transport systems in expression of the virulence (cariogenicity) of *Streptococcus mutans* are minimally defined *in vivo*, except for some preliminary reports (Tanzer *et al.*, unpublished observations), due in part to the multiplicity of those systems and their nominal functional redundancy (Slee and Tanzer, 1982; LeBlanc, 1994; Tao *et al.*, 1993). Further complicating this is the reported generation from the disaccharide sucrose of free glucose and fructose by extracellular invertase of *S. mutans* (Kuramitsu, 1973), although others have reported this activity to be intracellular among the mutans streptococci (Tanzer *et al.*, 1973, 1977). Also, extracellular fructosyltransferase and glucosyltransferases of the mutans streptococci most commonly colonizing humans, *S. mutans* and *S. sobrinus* (Coykendall, 1989; Tanzer *et al.*, 2001a), produce free glucose and free fructose, respectively, as by-products of polymer synthesis and thus available for transport into the cells (Tanzer *et al.*, 1985a; Kuramitsu, 1993; LeBlanc, 1994). Both of these transferases, as well as invertase, use dietary sucrose as substrate.

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Two of the genetic determinants of fructose transport are now known (Wen *et al.*, 2001); one of them also transports xylitol into *S. mutans*, which inhibits glycolytic metabolism (Trahan, 1995). Study of xylitol-non-transporting and thus xylitol-resistant (X^r) *S. mutans* thus provides opportunity for evaluation of the dimension of the role of fructose transport in virulence and the exploration of a possible basis of caries inhibition by xylitol.

Xylitol inhibition of caries has attracted considerable interest (Scheinin and Mäkinen, 1975; Mäkinen *et al.*, 1995; Isokangas *et al.*, 2000; Söderling *et al.*, 2000), as has the puzzling emergence, in the mouths of habitual xylitol users, of *S. mutans* that are resistant to metabolic inhibition by xylitol (X^r) (Trahan and Mouton, 1987).

S. mutans has at least three fructose transport systems, two of which are phosphoenolpyruvate (PEP)-dependent, fructose-specific, phosphotransferase systems (PTSs) that are encoded by two operons. Within them, the *fru*I gene is inducible by both fructose and sucrose. It encodes for a protein that transports fructose and xylitol. Its deletion renders the mutant cell's metabolism and growth resistant to xylitol inhibition (X^r). By contrast, *fru*CD gene is constitutive. It encodes for a protein that also transports fructose, but does not transport xylitol. *fru*CD deletion does not render the mutant cell's metabolism and growth resistant to xylitol (Wen *et al.*, 2001).

We have previously engineered, by allelic exchange, stable *S. mutans* isogenic deletion mutants of the sequenced wild-type strain UA159 (Ajdic *et al.*, 2002) that are defective in either or both of the two PEP-dependent fructose-specific PTSs (Wen *et al.*, 2001), and another stable isogenic mutant of UA159 that is defective in sucrose phosphorylase (*gtf*A$^-$) (Wen and Burne, 2001). This, and the availability of rats free of indigenous mutans streptococci, enabled the present research to aim to characterize the colonization and cariogenicity of fructose transport mutants of *S. mutans* strains that are either xylitol-sensitive (X^s) or X^r in the setting of a high-sucrose diet. It also provided us with an opportunity to gain insight about the counter-intuitive emergence of X^r *S. mutans* within the mouths of habitual xylitol users, a condition associated with caries inhibition (Trahan, 1995). We tested the hypothesis that fructose and xylitol transport deletions have no effect on colonization or cariogenicity in sucrose-fed rats.

MATERIALS & METHODS

Micro-organisms

Stable *S. mutans* isogenic deletion mutants of sequenced strain UA159 defective in the two genes encoding fructose PTSs, either *fru*I$^-$, *fru*CD$^-$, or both *fru*CD$^-$/*fru*I$^-$ (Wen *et al.*, 2001), and another stable isogenic mutant of UA159 defective in sucrose phosphorylase (*gtf*A$^-$) (Wen and Burne, 2001) were studied. The well-characterized virulent *S. mutans* strain NCTC-10449S (Tanzer, 1979; Tanzer *et al.*, 1985b) served as a positive control, and un-inoculated status a negative control for *in vivo* experiments. Phenotypes and genotypes of studied UA159 and its isogenic mutants were confirmed before and after *in vivo* experiments. Essential traits are described in the Table.

Animal Experiments

Breeders of rat strain TAN:SPFOM(OMASF)BR, maintained in a full-barrier facility, yielded progeny, born on the same day, for two *in vivo* experiments of identical design. The rats are free of indigenous mutans streptococci and amylase-binding streptococci (Tanzer *et al.*, 1985b, 2001b, 2003). Each experiment had 7 groups. In the first, there were 12 rats/group, while in the second there were 10 rats/group. Weanlings (21 days old) ate diet 2000 (56% confectioner's sugar [97% sucrose/3% cornstarch]) and drank sterile demineralized water, *ad libitum.* At 22 days of age, one group was inoculated with 10^9–10^{10} CFU of *S. mutans* wild-

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type UA159 or one of its isogenic mutants (Table), or with *S. mutans* 10449S. The infectious dose needed to colonize 100% of rats for mutans streptococci is about 10^5 to 10^6 cells. Each strain had an unambiguous antibiotic resistance phenotype. One rat group was not inoculated in each experiment. All animal procedures were approved by the IACUC of the fully accredited Univ. of Connecticut Health Center.

Animals' teeth were swabbed at 21 days after inoculation for recovery of flora, and, immediately after euthanasia, 42 days after inoculation, molars of one hemi-mandible were removed *en bloc* and sonified for recoveries of total flora (trypticase soy sheep's blood agar), total streptococci (mitis salivarius agar) (MS), and *S. mutans* inoculants (MS plus appropriate antibiotic). Recoveries were expressed in both relative counts (% of total recoverable flora) and absolute CFU counts/3 mandibular molars. Procedures for blinded scoring of caries lesions and statistical procedures have been detailed previously (Tanzer *et al.*, 1985b, 2001b).

RESULTS

Colonization and Recovery

Un-inoculated animals were again demonstrated to be free of mutans streptococci, and there was no evidence of cross-contamination among groups or of reversion of mutants to wild-type pheno- or genotype. All strains colonized well; mid-experiment tooth swab recovery data (not shown) were consistent with those at the date of death. Absolute count recoveries of inoculants after death from sonicates of 3 mandibular molar teeth (Fig. 1, panels A and C) were significantly lower (52 and 64%) for the *fru*I$^-$-inoculated group than from UA159- and all other mutant-inoculated groups, in both experiments ($p < 0.001$). Absolute counts from the *fru*CD$^-$-inoculated and the *fru*CD$^-$/*fru*I$^-$-inoculated groups were not consistently related to those from their WT-inoculated groups in the two experiments. Recoveries of the *gtf*A$^-$-inoculated groups were like those of the UA159-inoculated groups. In the second experiment, UA159 was recovered in higher absolute numbers than 10449S ($p = 0.020$).

The percentages of total recoverable flora from the molars represented by the inoculants (Fig. 1, panels B and D) were very high (80–93% of total recoverable flora) and not different from one another, except for those inoculated with the *fru*I$^-$mutant, which was significantly lower (42–52% of total recoverable flora) than other inoculated groups in the two experiments (both, $p < 0.001$).

Genotypes recovered from agar plates after sonification of molars were analyzed by PCR with gene-specific primers (Wen *et al.*, 2001), and results showed no detectable alterations in the respective loci (data not shown).

Caries Scores

The sum of smooth-surface and fissure scores for un-inoculated groups was much lower than for any mutans-inoculated groups (Fig. 2), indicating that the majority of caries lesions were attributable to *S. mutans* colonization ($p < 0.001$). The wild-type UA159 was less cariogenic than the internal control reference strain 10449S ($p < 0.001$ for the data pooled from the two studies, n = 22/group).

Enamel (E) caries scores for single and double fructose transport mutants were statistically lower for the *fru*CD$^-$-infected group than for the UA159-infected group; this was statistically supported only after the data from the two identical experiments were pooled ($p = 0.022$, n = 22/group). Enamel scores for the other groups infected with mutants of UA159 were not statistically significantly different from the UA159-infected groups, with or without pooling results from the two experiments.

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However, dentinal (Dm) scores for the *fru*I^{-}-infected group were 30–35% lower than those of the WT- or *gtf*A^{-}-infected groups (p = 0.011 and p = 0.004, respectively) when the data were pooled for the two trials (n = 22/group).

Infection by the *gtf*A^{-} mutant resulted in no loss of virulence expression on either the enamel or in the dentin.

DISCUSSION

Fructose transport *via* the two fructose-specific PTSs, singly or in concert, is at most a weak virulence determinant of inception of enamel caries lesions of rats eating a high-sucrose diet and is more clearly associated with the constitutively expressed *fru*CD gene than with the *fru*I gene. However, deletion of the fructose-and sucrose-inducible, xylitol-transporting, fructose-PTS encoded by *fru*I is associated with (1) diminished colonization on/in the teeth and (2) diminished ability to contribute to penetration of lesions into dentin. We cannot with certainty explain the failure of the double mutant *fru*CD^{-}/*fru*I^{-} to behave *in vivo* as simply the sum of behaviors of the *fru*CD^{-} and the *fru*I^{-} mutants. The possibility of pleiotropic effects increases with double mutants (Wen *et al.*, 2001), and it is evident that sugar metabolism is complexly regulated (Slee and Tanzer, 1982; LeBlanc, 1994; Cvitkovitch *et al.*, 1995).

It is perhaps not surprising that, in the face of a high-sucrose diet, the condition most associated with aggressive caries in humans and rats (Mandel, 1970; Tanzer, 1979; Tanzer *et al.*, 2001a), the impact of fructose transport *per se* is weak, given the recognition of multiple sucrose transport systems, transport of monosaccharides generated by extracellular glucosyl and fructosyl transferases, and putatively extracellular invertase, all producing fermentable carbohydrates that can be transported (Slee and Tanzer, 1982; LeBlanc, 1994), converted to intracellular polysaccharides available for subsequent catabolism (Birkhed and Tanzer, 1979) and directly catabolized glycolytically (Tanzer *et al.*, 1971; LeBlanc, 1994). However, the ability to discern the contributions of fructose transport systems *per se* has been enabled in this study by the availability of stable mutations in two fructose-specific transporters. It is unclear from these *in vivo* experiments whether the *fru*I deletion has in some way altered the ability of the mutant to grow in plaque biofilm, to adhere to the teeth in it, or both. *In vitro* growth rates of UA159 and its mutants studied here were similar (data not shown) to those previously reported (Wen *et al.*, 2001).

Notably, the now-extensive literature on xylitol inhibition of caries (Mäkinen *et al.*, 1995; Isokangas *et al.*, 2000), usually monitored by measure of frank cavitation of lesions into the dentin, is associated with what might seem to be paradoxical emergence among frequent users of xylitol of X^{r} *S. mutans* in the mouth (Trahan and Mouton, 1987). The present observations suggest that, in fact, X^{r} strains of *S. mutans* are of diminished virulence by virtue of compromised colonization of the teeth and compromised ability to induce lesions that penetrate dentin, *i.e*., to the point that they would have been scored in those clinical studies.

The present study also indicates that sucrose phosphorylase of *S. mutans* is not a virulence determinant in rats consuming a high-sucrose diet. This is in agreement with some authors (Barletta *et al.*, 1988), who used mono-associated gnotobiotic rats fed diet containing only 5% sucrose but 62% cornstarch, while in disagreement with others who repeatedly inoculated specific-pathogen-free rat dams and weanlings fed the same 56% sucrose diet as used in the present study, gave 10% sucrose to drink, and removed the major salivary glands of the weanling test animals (Yamashita *et al.*, 1993). It should also be noted, from the present data, that the now-popularly-studied sequenced strain UA159 (Ajdic *et al.*, 2002), while a very good colonizer of rats' teeth, nonetheless appears less cariogenic than NCTC-10449S. A cautionary note is thus in order concerning UA159's broad use in studies of pathogenesis. It may well be

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that UA159 has been maintained in laboratories on the bench-top or in incubators for years, during which it has undergone untold numbers of replications, perhaps selecting for diminished virulence. The microbiological literature is replete with data on loss of virulence of laboratory strains maintained in this way. Our laboratory strain NCTC-10449S, since the mid-1970s, has been maintained in a lyophilized or deep-frozen state. It is always retrieved from −70°C stocks prior to experiments.

In summary, in the presence of a high-sucrose diet, fructose transport *via* either or both of two fructose PTS mechanisms is a weak determinant of virulence on enamel, but the *fru*I-encoded PTS does contribute to the ability to colonize the teeth and induce lesions that penetrate dentin. To our knowledge, this is the first demonstration of dissociation of the impact of a strain of *S. mutans*' ability to induce decay of enamel *vs*. dentin.

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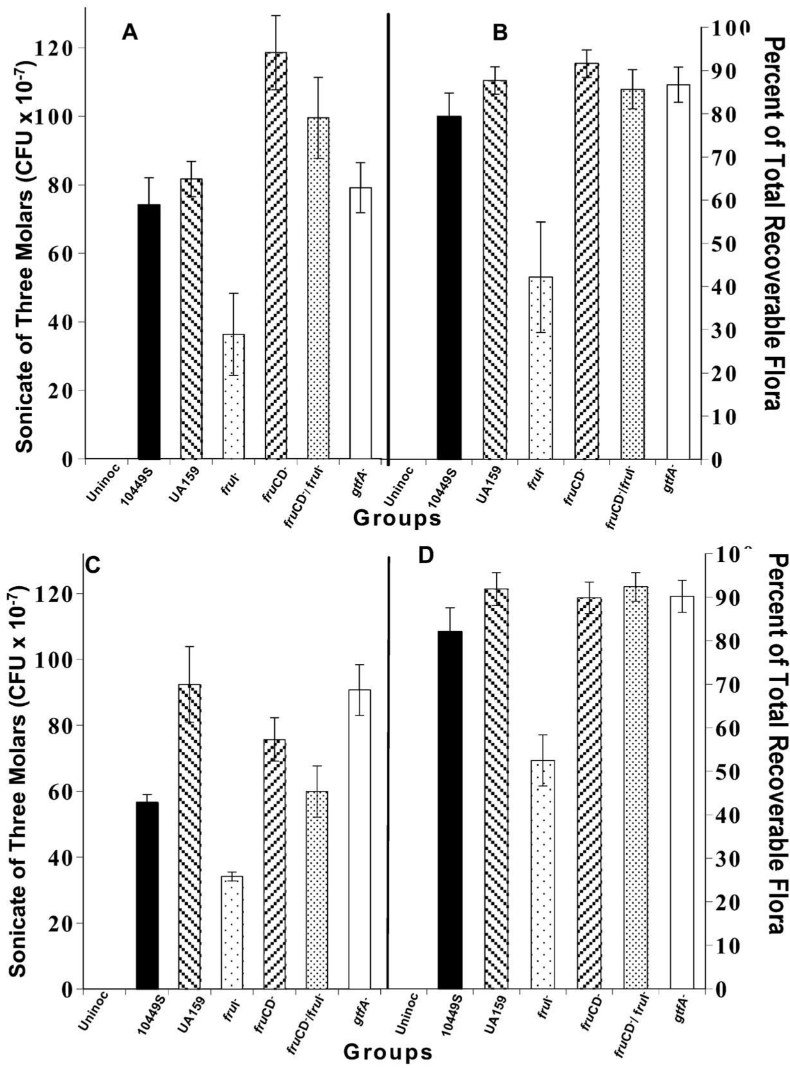

Figure 1.

Colonization of rats' teeth by designated *S. mutans* strains at 42 days post-inoculation. Strains are further described in the text and the Table. Two experiments are represented. In the first experiment (panels **A** and **B**), there were 12 rats *per* group; in the second experiment (panels **C** and **D**), there were 10 rats *per* group. Absolute counts are presented on the left, panels A and C, and percentages of total recoverable flora are on the right, panels B and D. All animals were free of indigenous mutans streptococci. The experimental design and microbiological methods are detailed in the text. Data are given as means ± SEM.

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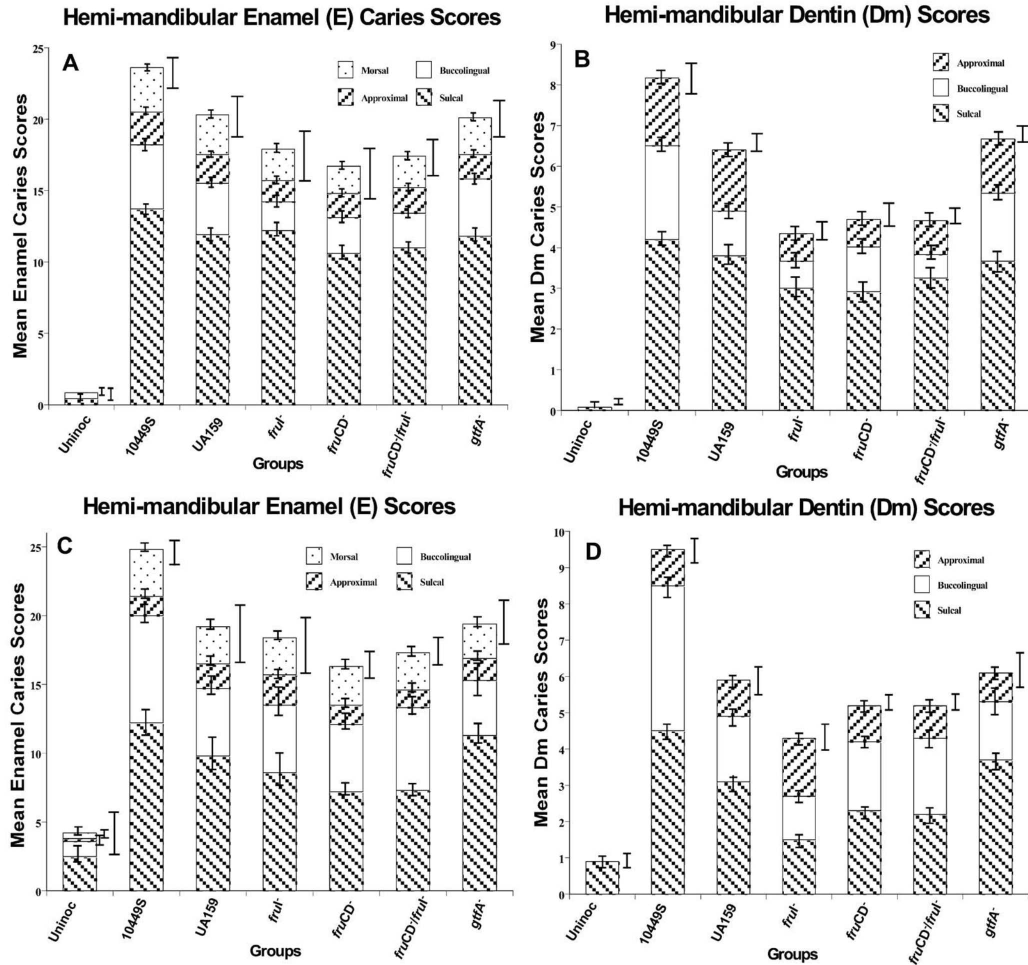

Figure 2.

Stacked-bar histogram of caries scores, according to tooth surface category, for the two experiments lasting 42 days post-inoculation with designated *S. mutans* strains. In the first experiment (panels **A** and **B**), there were 12 rats *per* group; in the second experiment (panels **C** and **D**), there were 10 rats *per* group. Mean ± SEM values are represented for each tooth surface category identified by bar shades, and the height of each stacked bar represents mean total caries score and its variance (SEM), indicated to its immediate right. Panels A and C depict the data for enamel (Keyes E) lesions, and panels B and D depict the data for dentinal (Keyes Dm) lesions, respectively. The scoring method of Keyes does not assess dentinal penetration on rat morsal tooth surfaces. The experimental design and blinded caries scoring methods are detailed in the text. The use of data pooled from the two experiments * indicates a statistically significant difference of the *fru*CD⁻ mutant-infected group for enamel caries scores by comparison with those of the wild-type UA159-infected group. ‡ indicates statistically significant differences of the *fru*I⁻ mutant-infected group for dentin scores by comparison with their respective wild-type UA159-infected and *gtf*A-infected group, as detailed in the RESULTS.

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Table

Strains Studied

Strains Used in This Study	**Gene Defect**	**Progenitor**	**Phenotype**	**References**
NCTC-10449S	Unknown	--	Known virulent, st^{r*}, X^s	Tanzer, 1979; Tanzer *et al.*, 1985b
UA159	Unknown	--	Known virulent, no antibiotic resistance, X^s	Ajdic *et al.*, 2002
TW17	*fru*I$^-$	UA159	Unknown virulence, em^r, X^r	Wen *et al.*, 2001
TW18	*fru*CD$^-$	UA159	Unknown virulence, tc^r, X^s	Wen *et al.*, 2001
TW20	*fru*CD$^-$/*fru*I$^-$	TW18	Unknown virulence, tc^r/em^r, X^r	Wen *et al.*, 2001
TW19	*gtf*A$^-$	UA159	Unknown virulence, km^r, X^s	Wen and Burne, 2001

* st^r, spontaneously streptomycin-resistant; em^r, erythromycin-resistant; tc^r, tetracycline-resistant; km^r, kanamycin-resistant; X^s, sensitive to inhibition of metabolism and growth by xylitol; X^r, resistant to inhibition of metabolism and growth by xylitol.